Immunolocalization of the apoptosis regulating proteins Bcl-2 and Bax in human endometrium and isolated peritoneal fluid macrophages in endometriosis

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Endometriosis, a debilitating disease associated with infertility, is characterized by the prolonged presence of ectopic endometrial tissue and the involvement of activated peritoneal fluid macrophages. Apoptosis, which occurs in both endometrium and peritoneal fluid macrophages, is controlled in part by members of the Bcl-2/Bax family of proteins. Here, through immunohistochemical staining, we investigated the Bcl-2/Bax status in endometrium and peritoneal fluid macrophages in endometriosis. Bcl-2/Bax immunoreactivity was found predominately in the glandular epithelial cells, mainly during the proliferative phase of the menstrual cycle for Bcl-2 but throughout the entire menstrual cycle for Bax. Ectopic endometrium contained a population of Bcl-2 positive, Bax negative tissue macrophages. Fluorescence-activated cell sorting of isolated peritoneal fluid macrophages showed that women with endometriosis had a significantly higher proportion of Bcl-2 positive macrophages than the non-endometriotic group. The proportion of Bax positive peritoneal fluid macrophages was significantly elevated in women without endometriosis. The increased proportion of Bcl-2 positive macrophages found in women with endometriosis may predispose these cells to resist apoptosis. The continued survival of these active cells could have important consequences for the survival and proliferation of the ectopic endometrial tissue.

Key words: apoptosis/Bax/Bcl-2/endometriosis/peritoneal fluid macrophages

Introduction

Endometriosis, the presence of endometrium outside of the uterine cavity, is a common disease, causing abdominal pain, dysmenorrhoea, dyspareunia and infertility in ~10% of the female population (Strathy et al., 1982). Its aetiology is unclear but it is thought to be due to the implantation and maintenance of disseminated uterine endometrium, predominantly on the ovary and pelvic peritoneum (Thomas and Prentice, 1992). The peritoneal cavity, the commonest site of endometriosis (Jenkins et al., 1986), contains fluid whose major cellular constituents are peritoneal macrophages (Eischen et al., 1994). In endometriosis, the number and secretory activity of these cells are increased (Halme et al., 1983, 1987) and recent evidence suggests that these cells play an important role in the development and maintenance of endometriosis (Ramsey, 1993; McLaren et al., 1996).

Endometrial cells (Hopwood and Levinson, 1976; Tabibzadeh et al., 1994) and peritoneal fluid macrophages (Albina et al., 1993; Reid et al., 1993), under the appropriate conditions, can undergo apoptosis. Apoptosis (programmed cell death) is critical to a number of physiological and pathological processes (Williams et al., 1994; Williams, 1994). It is distinct from cell death induced by toxic effects (necrosis) and is characterized by specific morphological features (Kerr et al., 1993). In the endometrium it is commonly observed in the glandular epithelium, and increases in frequency as the secretory phase of the menstrual cycle progresses, peaking during menstruation (Tabibzadeh et al., 1994). Although the precise regulation of apoptosis in this tissue is unclear, work in rabbit and human endometrium suggests that progesterone (Nawaz et al., 1987; Rotello et al., 1989) and tumour necrosis factor (TNF)-α (Tabibzadeh et al., 1995) are involved.

Activation of macrophages is critical for their host defence response (Adams and Hamilton, 1992). However, if not controlled these cells may damage the host by the unrestrained production of free radicals, lytic enzymes and cytokines (Nathan et al., 1980). Apoptosis is one mechanism which controls the activity of these cells (Munn et al., 1995). Therefore, any alterations in the response of the ectopic endometriotic tissue, and peritoneal fluid macrophages to apoptotic stimuli may have important implications for the survival of the ectopic tissue and the subsequent development of endometriosis.

Apoptosis is controlled by the expression of a number of regulatory genes, including c-myc, p53 and apo-1/fas (White, 1993; Osborne and Schwartz, 1994; Nagata and Golstein, 1995). However, one of the most important advances in our understanding of apoptotic cell death has come from studies of the oncogenic Bcl-2 family. Bcl-2 promotes cell survival by blocking apoptosis induced by a range of stimuli including growth factor withdrawal (Nunez and Clarke, 1994). Immuno-reactive Bcl-2 in endometrium is mainly localized in the glandular epithelium with maximal expression during the proliferative phase of the cycle, which gradually disappears or is markedly reduced during the secretory phase (Gompel et al., 1994; Otsuki et al., 1994; Koh et al., 1995). The disappearance of Bcl-2 coincides with the observed increase in the incidence of apoptosis during this phase of the cycle. It is now clear that the anti-apoptotic properties of Bcl-2 depend acutely on its
interaction with a number of potent antagonistic proteins (Bosie et al., 1993; Keifer et al., 1995; Yang et al., 1995). In particular, recent studies have shown that a related protein, Bax, antagonizes the survival-promoting activity of Bcl-2 (Oltvai et al., 1993). Bcl-2 needs to form heterodimers with Bax in order to function, and it is the ratio of Bcl-2 to Bax which predetermines the cells’ susceptibility to a given apoptotic stimulus (Oltvai and Korsmeyer, 1994).

The expression and localization of Bax in normal endometrium or eutopic and ectopic endometrium from women with endometriosis has not been described. In addition, the localization and expression of Bcl-2 and Bax in peritoneal fluid macrophages from normal women and women with endometriosis is unknown. Until the nature and extent of the interaction between these genes is known then their significance in the regulation of apoptosis in these tissues will remain unresolved.

We therefore investigated the expression of Bcl-2 and Bax in endometrium and isolated peritoneal fluid macrophages from women with and without endometriosis.

Materials and methods

Patient details

Women aged 24–44 years who were undergoing either diagnostic laparoscopy for pain, infertility or tubal ligation were included in this study. Endometriosis was diagnosed laparoscopically and confirmed histologically. Endometrium from women without endometriosis (n = 10) (normal endometrium) and eutopic endometrium (n = 10) (located within the uterine cavity) and paired ectopic endometrium (n = 10) (biopsied from the peritoneum) from women with endometriosis, throughout the menstrual cycle, were used in this study. Each group contained five proliferative and five secretory phase samples. Peritoneal fluid samples were collected from women with (n = 24) and without endometriosis (n = 24), throughout the menstrual cycle. Each group contained 12 proliferative phase and 12 secretory phase samples. The extent of endometriosis was determined according to the revised American Fertility Society scoring system (American Fertility Society, 1985). All patients included in this study had minimal to moderate endometriosis (scoring 1–12). This study was approved by the ethics committee of Addenbrookes NHS and written informed consent was obtained from each patient.

Isolation of peritoneal fluid macrophages

Peritoneal fluid was aspirated from the posterior cul-de-sac using a Veress needle. Macrophages were isolated by centrifugation through Ficoll-Hypaque 1077 (Sigma, Poole, UK) at room temperature for 10 min at 400 g. Cells were washed in phosphate-buffered saline (PBS) and counted before being resuspended at a concentration of 1×10⁶ cells/ml in PBS/1% bovine serum albumin (BSA). This method of isolation results in an enriched population of peritoneal fluid macrophages (>95%) which are not activated during the isolation procedure (Halme et al., 1983, 1987).

Collection of ectopic and eutopic endometrium

If the diagnosis of endometriosis was made, a second portal was inserted in the right iliac fossa through the rectus abdominus muscle lateral to the inferior epigastric vessels. A punch biopsy was obtained under direct vision on the video monitor. Eutopic tissue was obtained from the uterine cavity after gentle dilatation of the cervix to Hegar 8. Strips of endometrium were obtained by sharp curettage.

Immunohistochemical staining

Formalin-fixed paraffin-embedded sections of endometrium from women with and without endometriosis were de-paraffinized in xylene and hydrated gradually through graded alcohols. Antigenic unmasking was achieved by pressure cooking the slides for 1 min in 0.01 M sodium citrate. The slides were then incubated for 20 min in 10% goat serum to block non-specific binding, before incubation for 1 h with either a 50-fold diluted anti-human Bcl-2 mouse monoclonal antibody (Dako, Cambridge, UK) or a 500-fold diluted Bax anti-human rabbit polyclonal antibody (Santa Cruz Biotechnology, USA). Irrelevant isotype-matched mouse monoclonal antibody or peptide pre-absorption of rabbit polyclonal antibody were used as negative controls respectively. Endogenous peroxidase activity was inactivated by a 10 min incubation with 1% H₂O₂ in PBS. Binding was visualized following incubation with either a biotinylated goat anti-mouse antibody or biotinylated goat anti-rabbit antibody (1:200) for 1 h and subsequent complexing with avidin–biotin peroxidase (Vector, Peterborough, UK). The complex was detected with diaminobenzidine and hydrogen peroxide in 0.1 M Tris–HCl (pH 7.5).

Double immunofluorescence staining

Fluorescence double-staining for either Bcl-2 positive tissue macrophages or Bax positive tissue macrophages was carried out on all endometrial tissue sections. Sections were pre-treated as above, before incubating for 1 h in the dark with either a 10-fold diluted fluorescein isothiocyanate (FITC)-conjugated anti-human Bcl-2 antibody (Dako) or a 500-fold diluted anti-human Bax rabbit polyclonal antibody. Bax slides were then incubated for 1 h in the dark, with a 200-fold diluted FITC-conjugated anti-rabbit F(ab)₂ antibody fragment (Dako). Tissue macrophages were identified using phycoerythrin (PE)-conjugated macrophage marker Leu M3 (CD14). Slides were washed and mounted in aqueous mounting medium and viewed immediately. An irrelevant isotype-matched mouse monoclonal antibody or peptide pre-absorption of rabbit polyclonal antibody were used as negative controls respectively.

Flow cytometry

Peritoneal fluid macrophages were isolated, as previously described, and washed with PBS containing 2% fetal calf serum and adjusted to a concentration of 1–2×10⁶ cells/ml. The cells were first incubated with heat inactivated swine serum (56°C) for 20 min at room temperature to block non-specific Fc receptor binding. Following this the cells were fixed by incubating with 0.25% paraformaldehyde for 15–20 min in the dark at room temperature and permeabilized by incubating with 70% methanol for 60 min at 4°C. Cells were labelled for Bcl-2 with a 10-fold diluted FITC-conjugated anti-human Bcl-2 antibody and for Bax, the cells were labelled with a 500-fold diluted anti-human Bax rabbit polyclonal antibody followed by FITC-conjugated anti-rabbit F(ab)₂ antibody fragment. Macrophages were identified using the PE-conjugated macrophage marker Leu M3 (CD14). The negative control for Bcl-2 comprised cells treated with an isotype-matched control antibody. Controls for Bax consisted of the same polyclonal antibodies which had previously been pre-absorbed with control peptide. In addition, tubes containing only swine anti-rabbit-FITC F(ab)₂-labelled and an irrelevant directly PE-labelled monoclonal antibody were also used. The cells were then washed twice, resuspended in PBS and analysed using fluorescence-activated cell (FAC) sorting (Becton Dickinson) flow cytometer with Lysis II software (Becton Dickinson).

Statistical analysis

Statistical analysis was determined on original data from FAC-sorted peritoneal fluid macrophage samples. The data was not normally
Figure 1. Immunohistochemical staining for Bcl-2 and Bax protein in eutopic endometrium. (A) and (C) serial sections of proliferative phase endometrium stained for Bcl-2 and Bax protein respectively. (B) and (D) serial sections of secretory phase endometrium stained for Bcl-2 and Bax protein respectively. (E) and (F) serial section negative controls (sections stained using either an irrelevant mouse monoclonal or peptide pre-absorption of rabbit polyclonal antibody, in place of primary antibody at the same concentration). Original magnification $\times400$ in all cases. G = gland; BV = blood vessel. Arrow indicates a representative Bcl-2 positive lymphocyte.

Results

Immunohistochemical staining for Bcl-2

Eutopic and normal endometrium from women with or without endometriosis was characterized in the proliferative phase by strong immunoreactivity for Bcl-2 in the glandular epithelium which continued through into the early secretory phase of the cycle and was lost in samples obtained from the mid-late secretory phase (Figure 1A,B). Ectopic endometrium from women with endometriosis followed a similar pattern of staining to that found in eutopic tissue, with strong glandular immunoreactivity for Bcl-2 present during the proliferative phase, and little if any glandular staining during the mid- to late secretory phase (Figure 2A,B). Significant stromal staining for Bcl-2 was absent in all samples (Figures 1A,B and 2A,B) with the exception of lymphoid cells which were immunopositive in all endometrium samples stained, and throughout both phases of the menstrual cycle (Figures 1A,B and 2A,B). In paired eutopic endometrium and normal endometrium most of the Bcl-2 positive lymphoid cells were seen during the secretory phase. Endothelial cells in all samples were negative for Bcl-2, as was the surrounding vascular smooth muscle.

Bcl-2 positive tissue macrophages in ectopic endometrium

Staining for Bcl-2 in the ectopic endometrium from women with endometriosis revealed a population of Bcl-2 positive
cells which were morphologically uncharacteristic of lymphocytes. Following double fluorescence staining for both the macrophage marker Leu M3 (CD14) and the Bcl-2 protein it was demonstrated that these cells were Bcl-2 immunopositive macrophages (Figure 2E,F). Three out of the five proliferative and four out of the five secretory phase ectopic endometrium samples contained Bcl-2 positive macrophages, although the number of cells varied. Immunohistochemical staining for Bcl-2 in paired eutopic endometrium or endometrium from normal subjects failed to identify any significant population of Bcl-2 positive macrophages.

**Bcl-2 positive peritoneal fluid macrophages**

FAC sorting of peritoneal fluid macrophages for Bcl-2 protein demonstrated Bcl-2 positive macrophages in women with and without endometriosis. The overall proportion of Bcl-2 positive peritoneal fluid macrophages was significantly higher ($P < 0.05$) in women with endometriosis (median 45%, range 5–95%) compared with that seen in women without (median 14.5%, range 7–27%) (Figure 3). Women with endometriosis showed a cycle-dependent variation in the number of Bcl-2 positive macrophages, with significantly ($P < 0.01$) more Bcl-2 positive macrophages present in the proliferative phase (median 85.5%, range 40–95%) in comparison with the secretory phase of the cycle (median 17%, range 5–50%) (Figure 3). However, no cycle differences were seen in samples from women without endometriosis. The number of Bcl-2 positive peritoneal fluid macrophages found in the proliferative phase of the cycle in women with endometriosis was also significantly greater ($P < 0.01$) than that in both proliferative (median 14.5%, range 7–23%) and secretory (median 15%, range 7–25%) phases of the cycle from women without endometriosis (Figure 3).
macrophages in women without endometriosis (median 24%, range 12–42%) compared with those with endometriosis (median 12.5%, range 2–27%). No cycle-dependent variation in their number was seen (Figure 3).

**Discussion**

In this report we have compared for the first time the in-vivo distribution of both Bcl-2 and Bax using immunohistochemical staining in endometrium from normal subjects and eutopic/ectopic endometrium from women with pelvic endometriosis. We demonstrated a similar cyclical pattern of Bcl-2 immunoreactivity in the eutopic/ectopic epithelium to that previously described in normal endometrium (Gompel et al., 1994; Otsuki et al., 1994; Koh et al., 1995; Tabibzadeh et al., 1995) and adenomyotic tissue (Harada et al., 1996). However, these authors also reported a lack of Bcl-2 staining in ovarian endometriosis. This suggests pelvic endometriosis and adenomyosis may have similar Bcl-2 regulating characteristics to eutopic endometrial tissue, but different to those that exist in ovarian endometriosis. It may also indicate a difference in the pathogenesis between adenomyosis/pelvic endometriosis and ovarian endometriosis.

The persistent immunopositivity for the Bcl-2 antagonist protein, Bax, in the glandular epithelial cells of all endometrium throughout the menstrual cycle, implies that its expression is not hormonally regulated. Recent work by Teixeira et al. (1995) supports this, showing that Bcl-2 but not Bax expression is regulated by oestradiol in MCF-7 human breast cancer cell lines. Previous work has shown that the anti-apoptotic actions of Bcl-2 are acutely dependent on Bax and any imbalances will have a significant effect on the cells’ ability to survive an apoptotic stimulus (Oltvai et al., 1993). The ratio of Bcl-2 to Bax predetermines the cells’ susceptibility to a given apoptotic stimulus. Excess Bcl-2 will prevent the accumulation and dominance of Bax homodimers, leading to protection from a range of apoptotic stimuli. In situations where Bax predominates over Bcl-2 the formation of the Bax homodimers predisposes the cell to undergo apoptosis (Oltvai and Korsmeyer, 1994). However, it must be remembered that there are a number of other apoptosis/anti-apoptosis-inducing pathways which may also influence the susceptibility of the cells to undergo apoptosis (Vinatier et al., 1996).

The co-expression of Bcl-2 and Bax in the glandular epithelium during the proliferative phase of the cycle may explain the absence of apoptosis seen during this period. The persistence of Bax staining in the glandular epithelium, of both normal and eutopic/ectopic endometrium, in the absence of Bcl-2 in the secretory phase may predispose these cells to undergo apoptosis during this period. In addition, an increase in apoptosis in the glandular epithelium during the secretory phase of the cycle is well documented in normal endometrium (Tabibzadeh et al., 1994) along with increases in apoptosis-inducing cytokines such as TNF-α (Tabibzadeh et al., 1995). A similar lack of Bcl-2 staining was evident in ovarian endometriosis which also coincided with the appearance of apoptosis (Harada et al., 1996). However, apoptosis was not measured in our samples so the association between the lack
of Bcl-2, maintenance of Bax and the subsequent appearance of apoptosis was not confirmed.

Immunohistochemical staining revealed a population of Bcl-2-positive, Bax negative tissue macrophages present only in ectopic tissue during both phases of the cycle. The expression of Bcl-2 and absence of Bax may confer on these cells a decreased susceptibility to apoptosis, given the known properties of Bcl-2 and Bax, and may result in an extended life expectancy. Macrophages are highly active cells and in addition to their phagocytic and immunomodulatory properties can secrete an extensive range of growth and angiogenic factors (Sunderkotter et al., 1991; Adams and Hamilton, 1992). Therefore the presence of a population of potentially highly active macrophages, which are selected for survival, within the ectopic tissue may have important implications in pathophysiology of endometriosis. This takes on added importance since we have recently demonstrated, in ectopic endometrium, the presence of tissue macrophages which are immunopositive for the potent angiogenic growth factor, vascular endothelial growth factor (VEGF) (McLaren et al., 1996).

The majority of endometriotic tissue is bathed by peritoneal fluid whose main cellular constituent is peritoneal fluid macrophages (Eischen et al., 1994). These cells have been shown to secrete an extensive array of soluble factors including TNF-α (Halme, 1989), interleukin (IL)-1β (Polan et al., 1989), IL-1 (Simoni et al., 1994), IL-6 (Rier et al., 1994), interferon (INF)-γ (Keenan et al., 1994) and VEGF (McLaren et al., 1996) which may affect the growth and vascularization of tissue found within this region. In endometriosis there is an increase in number, activation status and secretory activity (Halme et al., 1983, 1987; Halme, 1989; Simoni et al., 1994) in these cells and their importance in the pathogenesis of endometriosis is now being realized (Ramsey, 1993; McLaren et al., 1996). Isolated peritoneal macrophage samples from women with endometriosis were found to contain a significantly higher proportion of Bcl-2 positive cells compared to women without endometriosis, with a reciprocal increase in the numbers of Bax positive cells in women without endometriosis. Activation of lymphocytes and macrophages often results in apoptosis, since prolongation of their enhanced activity would have detrimental effects (Cerami, 1992). In endometriosis we see an increase in the activation of peritoneal fluid macrophages. We speculate that the increased proportion of Bcl-2 positive peritoneal fluid macrophages in women with endometriosis results in an increased number of cells surviving the process of activation and thus delaying apoptosis, and in turn may explain the increased numbers of macrophages found in endometriosis. The presence of this increased population of active macrophages will have important consequences for the local peritoneal environment in which the endometriotic tissue finds itself.

Previous work with MCF-7 human breast cancer cell lines (Teixeira et al., 1995) and in normal endometrium (Otsuki et al., 1994) and also our results with eutopic and ectopic endometrium suggests that Bcl-2 expression may be regulated by steroid hormones. Cyclical variations in the proportions of Bcl-2 positive peritoneal fluid macrophages in women with endometriosis suggests that these cells may also respond to changes in the steroid environment. We have shown previously that peritoneal fluid macrophages contain mRNA encoding oestrogen and progesterone receptors, are also immunoreactive for these receptors and can respond to direct stimulation by steroids (McLaren et al., 1996). Thus steroids may directly regulate apoptosis in peritoneal fluid macrophages.

In summary, eutopic and ectopic endometrium are immunopositive for Bcl-2 and Bax in patients with endometriosis. The cyclical pattern of immunoreactivity for Bcl-2 is similar to that seen in endometrium from normal subjects and suggests regulation by ovarian steroids. Bax demonstrated no cyclical pattern of staining.

The presence of Bcl-2 positive Bax negative macrophages in endometriotic tissue gives rise to the possibility of a population of potentially highly active secretory cells, with reduced susceptibility to apoptosis, exerting their secretory effects within the ectopic tissue. The elevated population of Bcl-2 positive peritoneal macrophages in women with endometriosis may predispose these cells to survive activation and resist apoptosis. The continued survival of these activated cells would have important consequences for the survival and proliferation of endometriosis.

References


